Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens

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Summary: Cross-presentation involves the uptake and processing of exogenous antigens within the major histocompatibility complex (MHC) class I pathway. This process is primarily performed by dendritic cells (DCs), which are not a single cell type but may be divided into several distinct subsets. Those expressing CD8α together with CD205, found primarily in the T-cell areas of the spleen and lymph nodes, are the major subset responsible for cross-presenting cellular antigens. This ability is likely to be important for the generation of cytotoxic T-cell immunity to a variety of antigens, particularly those associated with viral infection, tumorigenesis, and DNA vaccination. At present, it is unclear whether the CD8α-expressing DC subset captures antigen directly from target cells or obtains it indirectly from intermediary DCs that traffic from peripheral sites. In this review, we examine the molecular basis for cross-presentation, discuss the role of DC subsets, and examine the contribution of this process to immunity, with some emphasis on DNA vaccination.

Introduction

The immune system consists of two major types of lymphoid tissues. Primary lymphoid organs like the thymus, which is responsible for generation of T cells from precursors and secondary lymphoid tissues such as the spleen, lymph nodes, and Peyer's patches, which represent sites of immune induction. Naïve T cells do not traffic through all parts of the body but simply recirculate between the different secondary lymphoid compartments, examining antigen-presenting cells (APCs) for the presence of their cognate ligand, in a passage that takes about one day to complete. To survey the entire body, T cells rely on the trafficking of antigen, either in free form or associated with APCs, from peripheral tissues via the lymphatics or blood to the secondary lymphoid organs. Each of these organs surveys different compartments of the body, with the spleen monitoring blood-borne antigens, while the lymph nodes screen their particular local regions.

Dendritic cells (DCs), which are an APC type found in virtually all tissues, appear to capture antigens, transport

them to the draining lymph nodes, and then present these antigens to naïve T cells. DCs possess the apparently unique ability to activate naïve T cells, causing their expansion and differentiation into effectors. Once they leave the lymph node, effector T cells are able to enter peripheral tissues, specifically targeting sites of inflammation where they perform their specific immune function. For cytotoxic T cells, this function could mean destroying either virus-infected cells or tumor cells or even APCs infected with intracellular parasites.

Cytotoxic T lymphocytes (CTLs) recognize antigens in the context of major histocompatibility complex (MHC) class I molecules. Two general mechanisms have been reported to contribute to how DCs process such antigens into this pathway for activation of naïve T cells. The first and simplest approach to access antigen to the MHC class I pathway within DCs is to directly express the antigen in these cells, either by infecting the cells, for example with virus, or transfecting DCs with DNA. Antigens synthesized within cells (endogenous antigens) are routinely processed into the MHC class I pathway, and there is no exception for DCs. Infection or transfection allows MHC class I-restricted antigen presentation. The second, less well-defined approach is for the DCs to capture antigens from another source and process these captured exogenous antigens into the MHC class I pathway. This form of presentation is referred to as cross-presentation. The type of antigens targeted for cross-presentation, the mechanism by which this presentation occurs, and the DC types that carry out this process are explored in this review with some focus on the role of cross-presentation in DNA vaccination.

What is cross-presentation?

Most cells have the capacity to present peptides on MHC class I molecules, a property that is particularly important for the identification of virus-infected cells by CTLs. Detection of virus-infected cells in this manner allows their destruction, thus limiting viral replication. To ensure only infected cells are killed, the MHC class I pathway within most cells is restricted to processing endogenously synthesized proteins for presentation, excluding exogenously derived proteins. As a consequence, cells infected with virus can present viral antigens on their MHC class I molecules and be identified for CTL destruction, but bystander cells that merely endocytose viral debris cannot process such antigens to form MHC class I-restricted complexes and are therefore not targeted.

For a few cell types, particularly DCs, the demarcation between endogenous and exogenous antigen is not as strict, and MHC class I-restricted presentation of both sources of antigen can occur. The unusual capacity to process exogenous antigens into the MHC class I pathway, referred to as 'cross-presentation', contrasts the 'direct' or 'classic' presentation route for endogenously synthesized proteins (Fig. 1).

The mechanistic basis for cross-presentation

The mechanisms underlying cross-presentation are only beginning to be elucidated. Three groups (1–3) recently report the existence of a process whereby phagosomes fuse with endoplasmic reticulum (ER)-derived vesicles. The resulting phagosome-ER hybrid compartment contains newly synthesized MHC class I molecules together with the components required for MHC class I peptide loading, such as the transporter associated with antigen presentation (TAP), tapasin, calreticulin, and ERp57 (4). Phagocytosed antigens are then transported to the cytosol adjacent to the phagosome by an as yet undefined mechanism that may involve the Sec 61 complex, a multimolecular channel that is normally used to transport secreted or membrane proteins into the ER and to 'dislocate' polypeptides from the ER to the cytosol (5–8). The exogenous antigens are then degraded by closely associated proteasomes, and the resulting peptides are transported back into the phagosome via the TAP complex for loading onto class I molecules (Fig. 1). When they reach the cell surface, the cross-presenting MHC class I molecules are endo-H sensitive as a consequence of trafficking directly from the ER without transiting through the Golgi, the usual route for the direct MHC class I presentation pathway (2). Lizée *et al.* (9) report that a tyrosine residue in the cytoplasmic tail of MHC class I heavy chains are critical for trafficking of these molecules to the phagosome and that substitution of this tyrosine with a phenylalanine leads to disruption of cross-presentation without affecting the endogenous presentation pathway.

Which cells cross-present?

The major cell type known for its capacity to cross-present antigens is the DC (10–14). These cells have no absolute defining characteristic, but they can be generally characterized as leukocytes that express the integrin CD11c and in their mature form express high levels of MHC class II, costimulatory molecules CD80 and CD86, are veiled or dendritic in appearance, and are able to initiate primary immune responses. DCs are not a single cell type but consist of several distinct subsets. As discussed later, only some of these subsets efficiently cross-present captured antigens.

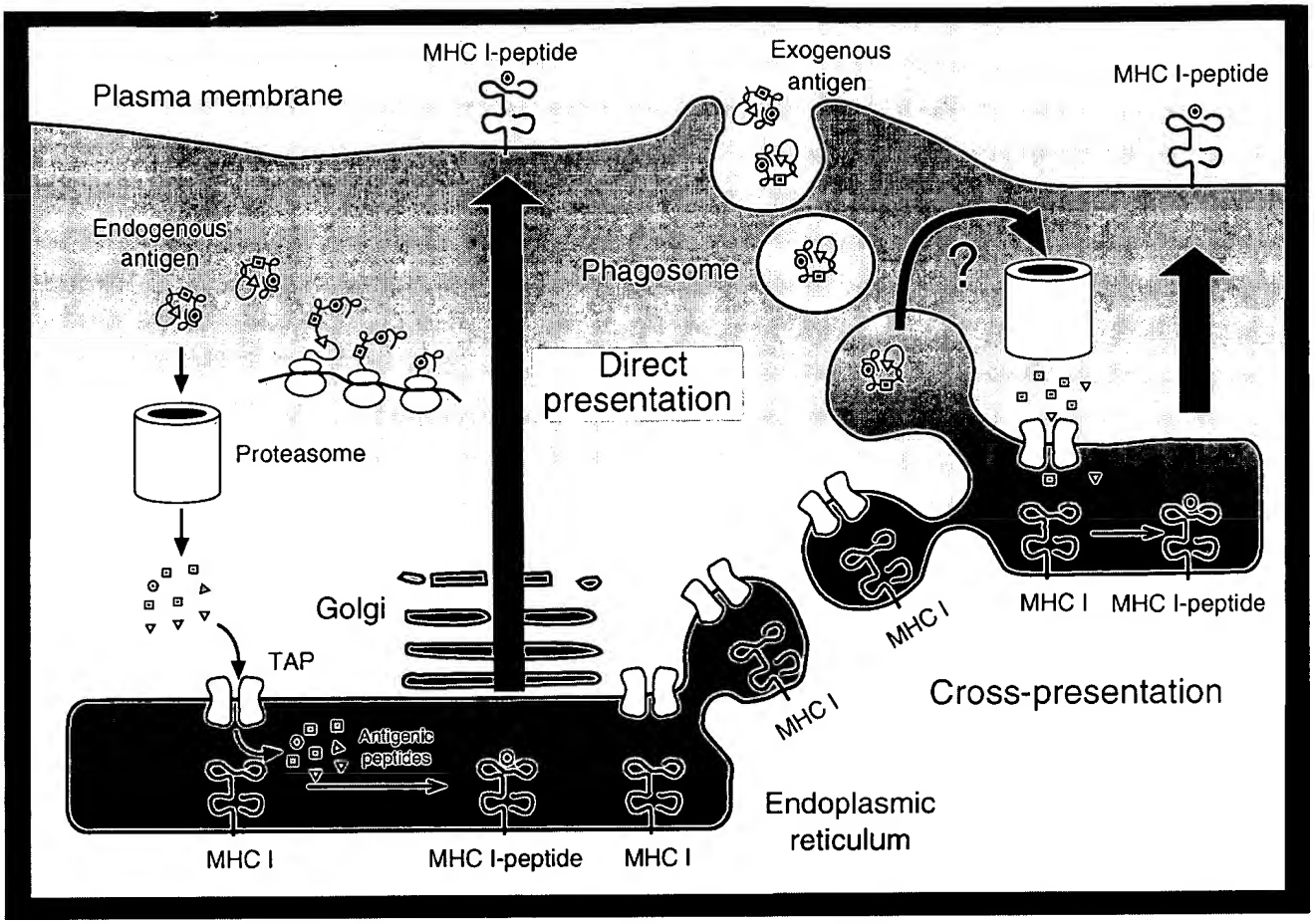

Fig. 1. **Major histocompatibility complex (MHC) class I presentation pathways.** Direct presentation involves the processing of endogenously synthesized antigens by the proteasome to break them down into peptides that are transported by the transporter associated with antigen presentation (TAP) complex into the endoplasmic reticulum (ER) for loading onto newly synthesized MHC class I molecules. These MHC class I molecules are then transported through the Golgi to the cell surface. Recent evidence (1–3) suggests that cross-presentation may involve the fusion of ER with early phagosomes to form organelles with all the required class I-processing machinery. Phagocytosed proteins are retrotransported out of the phagosome to be degraded by closely associated proteasomes. Peptides are then transported back into the phagosome by the TAP complex and loaded onto newly formed, or perhaps recycled, MHC class I molecules for transport to the cell surface.

Several other cell types have been reported to cross-present, including B cells (15), endothelial cells (16, 17), and particularly macrophages (18–22), the latter of which have in some cases been compared to DCs (22). Endothelial cell types shown to cross-present antigens include liver sinusoidal endothelial cells (16), which appear to cross-present food antigens for tolerance induction, and endothelial cells surrounding islets of Langerhans (17), which may perform this function to allow specific effector CTLs to target this organ. While cross-presentation by B cells and even endothelial cells might need further validation, a vast array of evidence supports this function in both DCs and macrophages.

What antigens are cross-presented?

Several types of antigens have been reported to be cross-presented. These include soluble proteins (12, 23), immune complexes (13, 24), intracellular bacteria (25), parasites (26), and, most importantly, cellular antigens (22, 27–34). Of the latter, various cell types have been shown to be cross-presented including virus-infected cells (22, 27,28), transfected tumors (29, 30), various normal tissue cells, like spleen cells (31, 32), islet β cells (33, 34), and proximal tubular cells of the kidney (33), and even protein-coated cells (31, 35). Cellular antigens were the first type of antigen reported to undergo cross-presentation in studies where mice were immunized with allogeneic cells and examined for the MHC restriction of the subsequently induced CTL response (32). Such immunization generated minor histocompatibility antigen-specific CTLs that were restricted to both donor and, surprisingly, host MHC class I molecules. The latter could only occur if host APCs had captured donor cellular antigens and processed them into the MHC class I pathway for stimulation of CTL

immunity. This ability originally led to the term cross-priming, because antigens crossed over from the donor cell to the host APC, which then initiated MHC class I-restricted CTL priming. The capacity to induce such responses to cellular antigens was later proposed to relate to a need to generate CTL immunity to virus-infected cells, particularly for tissue-specific viruses (36). Without cross-priming, viruses could escape immune recognition by avoiding the infection of DCs, since presentation by DCs appears essential to initiate immunity. In other words, if direct presentation (*Fig.* 1) was the only way to introduce antigens into the MHC class I pathway, then DCs (which initiate immunity) would need to be infected with a virus for priming to occur. If this case were true, smart viruses could escape CTL immunity by avoiding the infection of DCs. Cross-priming prevents this escape mechanism by allowing DCs to sample antigens from other cells and present them in the MHC class I pathway. Cross-priming also takes care of another major virus escape mechanism, down-regulation of antigen presentation. Many viruses possess means to impair or block the direct pathway of MHC class I-restricted presentation (37–41), most likely to provide some protection against CTL lysis of virus-infected cells. The ability to cross-prime, which is unlikely to be affected by such blocking mechanisms, provides DCs with the capacity to initiate effective CTL immunity in the face of viral mechanisms that block direct presentation. This process would also obviously be beneficial in the case of viruses that inhibit other DC functions, such as maturation, or for those viruses that rapidly kill the cells they infect.

Access to the cross-presentation pathway

One question for which the answer is currently not fully elucidated is how cellular antigens enter the cross-presentation pathway. Recent studies have implicated phagocytosis in this process (1–3), but several mechanisms have been speculated to lead to antigen capture, including uptake of apoptotic cells (10), nibbling of live cellular material (42, 43), transfer of heat shock proteins (HSPs) (44), and exosome uptake (45, 46). At present, there is limited *in vivo* evidence to categorically implicate any of these mechanisms. Capture of apoptotic cells by DCs has received a great deal of emphasis (10, 14, 47–50), with the prevailing but most likely false impression being that apoptosis is essential for entry of cellular material into the cross-presentation pathway. It is clear that apoptotic material can be cross-presented, as shown by the *in vitro* cross-presentation of apoptotic influenza-infected macrophages by human DCs (10) or by the *in vivo* induction of cross-presentation after islet damage (51). However, it is far from certain that apoptosis is essential for access of antigen to the cross-presentation pathway. In fact, there is some evidence that live cellular material may be sampled for this process (42, 43). Barratt-Boyes and colleagues (42) showed that monkey DCs are able to capture material from other cells in a nibbling process that does not require apoptosis of the donor population and appears to be mediated via a class A scavenger receptor (43). These data are derived from *in vitro* studies, however, and the role of such a mechanism *in vivo* is yet to be demonstrated. Sigal's group (22) has also provided evidence that live cellular material can be cross-presented.

Several studies using DNA vaccination have employed strategies to enhance priming by specifically inducing apoptosis of transfected cells (52, 53). While this can improve priming under the defined circumstances described, it is unclear whether apoptosis mediates antigen uptake or simply enhances the level of antigen presentation, because more antigen is available upon the death of those cells synthesizing the DNA-encoded antigen. In other words, logic would dictate that, at least in the short term, more antigen will be available for cross-priming if the cells expressing it die, because none will be retained by the donor cells. So, induction of apoptosis may be effective in enhancing cross-priming under some circumstances, but whether it is essential must await more definitive studies.

As a third mechanism for antigen access to the cross-presentation pathway, it is worth mentioning that several studies have illustrated the capacity of HSPs to efficiently prime CTL immunity (44). These proteins are normally involved in protein folding and stability, but when isolated from cells, HSPs may be used as a source of antigen that can facilitate CTL priming. Specific receptor-mediated mechanisms exist for the capture and internalization of HSPs by DCs (54, 55), suggesting that cross-presentation of HSP-derived antigenic determinants is a legitimate mechanism for cross-priming by DCs. However, no evidence is available to indicate that HSPs are generated and released *in vivo* for the specific purpose of donating antigen to the cross-presentation pathway. Thus, whether this pathway is an artefact of experimental design or an authentic *in vivo* surveillance mechanism is yet to be clarified.

Exosomes, which are small membrane-encased vesicles released by many cell types (56), have been reported to act as a source of cross-presented antigens (45, 46) and in some cases of whole peptide-loaded MHC molecules (56). Like HSPs, DCs appear to capture exosomes and may cross-present the proteins contained within them, but whether such presentation normally occurs *in vivo* or is again an *in vitro* artefact of experimental design has not been determined. Considering the efficiency with which both exosomes and HSPs are able to

prime CTL immunity', it is hard to imagine that they do not play a role in some forms of cross-priming. Doubt over this possibility must remain, however, because hard experimental evidence is lacking.

While cellular antigens are an obvious target for cross-presentation, soluble antigens (12, 23) and even immune complexes (13, 24) can also access this pathway. Examination of the efficiency of cross-presentation of soluble versus cell-associated ovalbumin (OVA) *in vivo* shows that after intravenous administration, cellular OVA is cross-presented with 50 000-fold more efficiency than soluble OVA (57). This finding suggests that access of soluble proteins to this pathway is poor, but it is also possible that soluble OVA is quickly eliminated by serum proteases or sequestered from the medium by active uptake or adherence to bystander cells. The fact that presentation of OVA via MHC class II requires a concentration 500-fold higher when the antigen is soluble than when it is cell-associated supports this alternative explanation. Perhaps such processing only targets local high concentrations of soluble proteins, as might occur during release of virions from cells infected with lytic viruses. In contrast to soluble antigen, immune complexes appear to be efficiently cross-presented (24), and they are possibly important in the induction of rapid secondary responses to intracellular pathogens for which antibody responses have been previously generated.

It is possible that yet other receptor-mediated mechanisms may exist that enable entry of antigen into the cross-presentation pathway. Interestingly, while some types of DCs are unable to cross-present soluble OVA when exposed *in vitro* (Behrens *et al.* manuscript submitted), these same DCs cross-present OVA when injected *in vivo* (12). This outcome might be explained by the ability of mannose-binding lectin, or low affinity T-independent antibodies, or even complement to target soluble OVA *in vivo* for access to the cross-presentation pathway of particular DC types.

Regulation of cross-presentation

There is limited knowledge regarding the regulation of cross-presentation at the cellular or molecular level. Some cells, including certain subsets of DCs (11, 12, 14, 58) and endothelial cells (17), appear to constitutively cross-present antigens, while other DC types require activation by stimuli such as immune complexes (13), T-cell help (59), toll-like receptor ligands (60), or other undefined signals (61). This area of study is emerging and suggests the cross-presenting capacity of DC may in many cases be regulated to occur only under specific circumstances. Why this regulation is necessary and how it contributes to immunity must await further efforts.

What type of responses involve cross-presentation?

We have discussed the nature of antigens that are cross-presented without saying much about the type of responses that involve this process. In some types of immunity, it appears relatively straightforward to implicate a role for cross-presentation, but in others a decisive role can only be speculated, with the contribution of cross-presentation and direct presentation difficult to separate. In the first category, responses to minor histocompatibility antigens, as first described for cross-priming, clearly occur by this process, because resulting CTL immunity is restricted to host MHC class I molecules. Similarly, CTL induction to protein-coated cells, where donor cells do not express MHC class I, also obviously involves cross-presentation. The generation of MHC class I-restricted responses to intracellular bacteria (25) and parasites (26) may also involve access to the cross-presentation pathway, especially for those organisms that survive in endocytic vesicles of DCs and macrophages. In fact, it might be argued that the cross-presentation mechanism was originally selected to control such intracellular pathogens, because this pathway is important to target infected DCs and macrophages for lysis by effector CTL.

There are four areas where the involvement of cross-presentation is controversial: tumor immunity, self-tolerance, viral immunity, and DNA vaccination. Three of these are briefly discussed immediately below, but DNA vaccination is covered in more depth later in this review. For tumor immunity and self-tolerance, there are clear examples where cross-presentation is utilized, so the issue is really whether cross-presentation is 'normally' used or is only observable in defined experimental models. Pardoll's group (29, 30) clearly implicated cross-priming when they reported that CTLs induced in a tumor cell line transfected with the gene for influenza nucleoprotein (NP) were restricted to the MHC class I haplotype of cells of the host's bone marrow compartment, which were also required to express TAP. Many years earlier, Gooding and Edwards (27) had similarly shown that SV40-transformed tumor cells generated SV40-specific CTLs restricted to the MHC haplotype of the host, implicating cross-priming.

For self-tolerance, several studies have shown that expression of model self-antigens under the control of the rat insulin promoter (RIP) leads to cross-presentation of these antigens by host bone marrow-derived DCs (34, 62–64). This form of cross-presentation causes deletion of self-reactive CTLs in a process referred to as cross-tolerance (62, 65).

While definitive examples of cross-presentation exist for many tumors and for self-tolerance, there are equally numerous

examples where such antigens are apparently not cross-presented. Bevan (32) first reported that minor histocompatibility antigens expressed by the P815 mastocytoma did not cross-prime. Kundig *et al.* (66) later reported that MC57 cells transfected with an antigen from lymphocytic choriomeningitis virus (LCMV) did not cross-prime immunity to this antigen. In an extensive series of studies, Zinkernagel's group (67) recently examined several different tumors transfected with LCMV and vesicular stomatitis virus (VSV) antigens, showing that these tumor cells only induced CTLs by direct presentation, and they failed to generate immunity by cross-priming. Similarly, Ohashi *et al.* (68) showed that mice expressing an LCMV protein under the control of the rat insulin promoter (RIP) did not cross-present this antigen but instead their CTLs ignored it unless first activated by viral infection after which they were able to destroy the islet tissue. Failure to demonstrate cross-presentation under all circumstances prompted critics to question whether cross-presentation is merely an artifact of certain models. Certainly, cross-presentation is not only induced to model antigens, because the original definition of cross-priming arose as a result of induction of CTL immunity to minor histocompatibility antigens expressed by normal cells (69). Thus, perhaps the question might be better asked in reverse, i.e. whether an absolute lack of detection of cross-priming for certain antigens might be due to the nature of these antigens. In other words, perhaps some systems cannot measure a component of cross-priming, because the antigens they use are resistant to this pathway. On this point, numerous studies that failed to detect cross-presentation to self- or tumor antigens almost invariably involved the use of proteins from LCMV. Perhaps this virus has developed proteins that intentionally subvert cross-presentation. In support of this view, spleen cells from transgenic mice expressing glycoprotein of LCMV in all tissues could not generate CTL immunity by cross-priming when injected into normal mice (67). As this robust and traditional approach has been used extensively to generate cross-priming to minor histocompatibility antigens by many groups and to single proteins such as OVA or β-galactosidase loaded within or expressed by spleen cells (31, 57), failure of the LCMV antigens to achieve similar cross-priming must raise some questions. Perhaps, we should consider more informative those studies that have used antigens never subjected to selective pressure by cross-presentation, e.g. OVA, β-gal, and yellow fluorescent protein, all of which are cross-presented (31, 64).

Is cross-priming involved in viral immunity?

Viruses are intracellular pathogens that utilize host-cell protein synthesis machinery to replicate. These organisms are possibly the major reason for the classical or direct MHC class I presentation pathway, which presents endogenously synthesized antigens to allow targeting of virus-infected cells for destruction by CTLs. To generate effective CTL immunity, however, naïve CD8 T cells must be primed to antigens presented on DCs (70–72), which must either be infected with virus or else cross-present viral antigens from other virus-infected cells. Cross-priming has been theorized to be important for immunity to tissue-specific viruses that do not infect DCs (36), and it may also be crucial for priming CTLs to viruses that can impair the immune function of DCs they infect (73). The difficulty in determining the role of cross-presentation in viral immunity arises when we try to assess its contribution relative to that of direct priming by infected DCs. Several groups have shown that virus-infected cells in various forms can be cross-presented *in vitro* (10, 48) or can cross-prime *in vivo*, resulting in the generation of CTL responses to viral proteins (22, 27, 29, 74). In addition, detection of priming in the face of virus-encoded mechanisms that impair direct presentation has implicated a component of cross-priming in viral immunity (75, 76).

To study the potential of cross-priming in viral immunity, Prasad *et al.* (74) generated adenovirus vectors encoding the influenza virus NP under three different promoters: one specific for the lung, one for the skin, and a ubiquitous promoter. When these recombinant viruses were administered by various routes, priming to NP occurred for all routes when driven by the ubiquitous promoter, but only primed during lung administration when using the lung-specific promoter, and only by the skin when driven by the keratinocyte promoter. These observations are most easily explained by the cross-presentation of tissue-specific virally expressed antigens.

The same group (76) examined CTL priming by vaccinia virus when direct presentation was inhibited by recombinant expression of US2 or US11 proteins from human cytomegalovirus. These proteins cause the degradation of newly synthesized MHC class I molecules, thus inhibiting class I-restricted presentation by those cells infected by the virus. This study revealed that CTL activity was reduced 25–50%, suggesting that at least this portion of CTL activity was generated by direct priming and that the remaining portion may be largely due to cross-priming. The contribution of cross-priming was further implicated when the CTL repertoire was examined. Some specificities were not induced by US2 and US11 recombinant vaccinia, and these same specificities were absent when virus-infected HeLa cells were used specifically to cross-prime. This study also suggested that the repertoire of CTLs induced by direct and cross-priming is not completely overlapping.

One of the best demonstrations that cross-priming can facilitate CTL immunity during actual virus infection was reported by Sigal et al. (28). These authors transgenically expressed the human cellular receptor for poliovirus in mice, which normally lack this receptor and are therefore not able to be infected by this virus. When CTL immunity was examined in chimeric mice that only expressed the poliovirus receptor on non-bone marrow-derived cells, efficient priming of CTLs to viral antigens was shown to occur via presentation by bone marrow-derived cells. This priming, they concluded, could only occur if host DCs captured viral antigens from infected non-bone marrow-derived cells, i.e. by cross-priming.

More recently, it was shown that trafficking of MHC class I molecules to the phagosome directly from the ER required a specific signal sequence in the cytoplasmic tail of the MHC class I heavy chain (9). When this sequence was mutated, normal peptide loading in the ER and traffic of MHC class I peptide complexes through the Golgi was maintained, leaving direct presentation intact. However, the mutated MHC class I molecule was deficient at cross-presentation, presumably due to impaired transport to the ER-phagosome compartment for cross-presentation. Transgenic mice expressing this form of mutant MHC class I molecule were examined for their response to VSV and Sendai virus to assess the contribution of cross-priming. In this case, CTL responses were reduced by about ninefold, though quantitation was imprecise. This finding implied that cross-priming is important in CTL priming to these viruses, but it left open the question of whether the residual priming was due to antigen processed by the direct MHC class I pathway in infected DCs or to a failure to totally impair cross-presentation by this mutation.

Because of the difficulties associated with identifying the contribution of cross-priming versus direct priming in an unmanipulated virus-infected mouse, we have taken the alternative approach of identifying those DC subsets that present viral antigens during infection, with the idea that such knowledge may lead to identification of the antigen presentation mechanism(s) involved. Analysis of the DC subsets involved in priming CTL immunity to various viral infections, including herpes simplex virus (HSV) type 1, influenza virus, and vaccinia virus, has shown that a single subset, which express CD8α and CD205 (see below), are the main contributors to activation of naïve virus-specific CTLs (71, 77, 78). As we discuss below, these DCs are also the major subset responsible for cross-presentation (11, 12, 64), providing a tantalizing though unproven link between cross-presentation and viral immunity.

Murine DC subsets

To fully understand the mechanisms underlying CTL priming to cellular antigens, whether viral, DNA-encoded, or of tumor origin, it is important to identify the APC type responsible for initiating this immunity. Several studies have shown that antigen presentation by bone marrow-derived cells (31, 79, 80) or $CD11c^+$ cells (70, 71) is important for responses to pathogens and other antigens, implicating DCs in this process. In fact, DCs are generally regarded as the only cell type capable of priming naïve T cells (81).

However, DCs are heterogeneous (82, 83), and to fully elucidate the mechanistic basis of priming, it is necessary to identify the precise subset(s) involved in presentation of each antigen or pathogen type. While it may be tempting for the reader to skip over our description of DC subsets with the view that it is complicated, poorly defined, or inconsistent, we encourage you to press on, as recent studies, particularly by Shortman and colleagues (84–86), have begun to provide a consistent and reproducible ability to identify at least six different subsets of DCs. Furthermore, understanding why there are so many different subsets is key to deciphering the induction phase of immunity. Based on incorporation of bromodeoxyuridine (BrdU) and several other findings (86–88), the six murine DC subsets that we will describe here do not appear to be precursor-product related but represent distinct sublineages.

Phenotypic markers for differentiation of DC subsets

The first major subdivision of DCs is between plasmacytoid and conventional subsets (*Table 1*). This subdivision was originally made in the human (89) and has only recently been extended to the mouse (86, 90, 91). There is little evidence that plasmacytoid DCs are important for antigen presentation in the induction phase of immunity, and their key property seems to be production of interferon αβ (IFNαβ) (92, 93). This ability is clearly important for blocking viral replication, but it has also been reported to activate other DCs, including those involved in cross-priming (94). Plasmacytoid DCs differ from conventional DCs in the mouse in that they express CD45RA and have a lower level of CD11c (86).

The remaining five subsets of DCs, which we call conventional DCs, can most easily be categorized by first dividing them into two groups: the blood-derived DCs, of which there are three, and the tissue-derived DCs, the remaining two (*Table 1*). All three conventional DC subsets in the spleen

Table 1. DC subsets, surface phenotype, and some important properties

DC type	Surface phenotype						Derivation	Distinguishing properties
	CD11c	CD8	CD4	CD205	CD11b	CD45RA		
CD8 DC	+	+	–	+	–	–	Blood	High IL-12 Cross-presentation of cellular antigen Cross-priming Cross-tolerance
CD4 DC	+	–	+	–	+	–	Blood	Most numerous DCs in spleen
$CD4^-CD8^-$ DC	+	–	–	–	+	–	Blood	High IFN-γ
Langerhans' cell	+	–/low	–	Very high	+	–	Skin epithelia	Traffic to lymph node from skin Present contact sensitizing antigens
Dermal/interstitial DC	+	–	–	+	±	–	Tissue	In all tissues Traffic to draining lymph nodes Prime CD4 T-cell immunity to tissue infections
Plasmacytoid DC	Low	±	±	–	–	+	Blood/tissues	High IFN-α, do not look like DCs until stimulated

DC, dendritic cell; IFN, interferon; IL-12, interleukin-12.

(84, 95) are blood-derived, because there is no lymphatic drainage to this organ. Whether they reach the spleen as precursors or as fully differentiated DCs is not yet clear. The same three subsets of blood-derived DCs found in the spleen are also present in the lymph nodes (85), but in this site there are also the progeny of tissue-derived DCs. Lymph nodes draining the skin contain two tissue-derived DC subsets. These are the Langerhans' cells and the dermal or 'interstitial' DCs (*Table 1*). Because organs other than the skin do not contain Langerhans' cells, non-cutaneous lymph nodes will not contain this DC type, but these organs contain their own interstitial DCs, which in general appear similar to the dermal DCs. Unfortunately, we must confuse the issue slightly, by raising the possibility that yet other DC subsets exist that are specialized for particular organs (raising the total number of subsets above six), but this discussion is beyond the scope of this review (Belz *et al.* manuscript submitted).

Blood-derived conventional DCs found in the spleen and lymph nodes can be separated into three subsets based on expression of CD4 and CD8α (84, 95). One subset expresses only CD4 (CD4 DC), another only CD8α (CD8 DC), and the third expresses neither of these markers ($CD4^-CD8^-$ DC). Other useful markers are listed in *Table 1*. It should be noted that plasmacytoid DCs may also express CD8α, especially upon activation (85), but as stated above, plasmacytoid DCs can be easily distinguished from conventional CD8 DCs by their expression of CD45RA. We need to stress here that our definition that these three subsets are blood-derived only strictly applies to the spleen, and it is possible, though unlikely, that in the lymph nodes any of these subsets may have trafficked through the tissues to reach this site. This definition is really designed to provide a simple subdivision.

The two clearly tissue-derived DC subsets, the Langerhans' cells and the dermal DCs, can be distinguished from most blood-derived DC by their expression of CD205 (85), although this marker is also expressed on CD8 DCs. They can be distinguished from this latter subset by CD8 and CD11b expression, because tissue-derived DCs are $CD8\alpha^{low/-}$ $CD11b^+$, while CD8 DCs show the reciprocal phenotype, $CD8^+CD11b^-$. There is some evidence that CD8α may be upregulated a little on Langerhans' cells as they move to the draining lymph node (LN), but this level is minimal and much lower than on CD8 DC (77). Finally, Langerhans' cells and dermal DCs are most easily distinguished from each other by their respective expression of high versus intermediate levels of CD205, and further by the high intracellular expression of langerin in Langerhans' cells (96). It should be noted with some caution that CD8 DCs express moderate levels of langerin, so this is not a marker unique to Langerhans' cells, though expression by Langerhans' cells is distinctly higher than that of CD8 DCs.

In summary, there are six major subsets of DCs (*Table 1*), which may be divided into the plasmacytoid DC and five subsets of conventional DCs. These five latter subsets consist of three blood-derived subsets (CD4 DC, CD8 DC, and $CD4^-CD8^-$ DC) and two tissue-derived subsets (Langerhans' cells and dermal/interstitial DCs), all of which appear to be distinct sublineages and not precursor-product related.

Some known functions of the different DC subsets

Considering the enormous research effort surrounding DCs, there is limited knowledge of the precise function of individual subsets. This lack of information is partly responsible for

the view that subset division of DCs may be largely academic. However, as information accumulates, distinct roles are beginning to emerge for individual DC populations.

Plasmacytoid DCs are able to produce large amounts of IFNαβ upon encounter with various stimuli, particularly toll-like receptor (TLR) ligands, such as CpG, or through detection of virions (90, 91, 97). The exact role for plasmacytoid-derived IFNαβ in immunity is not yet clear but may contribute to controlling viral replication or to the activation of other DCs, such as those responsible for cross-priming (94). Other DCs may also produce large amounts of IFNαβ, but this production appears to depend on viral infection of the DCs and is not stimulated simply by virions (93).

Langerhans' cells are probably one of the best-studied DC subsets (98). These cells reside in the skin epithelia, where they are thought to act as sentinels for the detection of invading pathogens. Pathogen encounter is proposed to induce migration of Langerhans' cells to draining lymph nodes, where they then prime T-cell immunity. The ability of these cells to migrate to the lymph nodes and induce immunity has largely been derived from studies using skin-sensitizing agents such as fluorescein isothiocyanate (FITC) (99), with few studies strongly implicating a role for Langerhans' cells in response to true pathogen infections. While it is clear that these cells migrate from the skin to the lymph nodes, there is largely only circumstantial evidence that migrating Langerhans' cells are directly responsible for antigen presentation for priming of T-cell immunity to antigens captured in the skin. In most cases, adoptive transfer of DCs labeled with skin-sensitizing agents has not precisely identified the priming cells as Langerhans' cells. In a recent article, Jacob and associates (100) used an ingenious system to track DCs that were transfected with DNA via gene gun administration. Recipient mice were transgenic for the *lacZ* gene, which encodes β-galactosidase, but in this case *lacZ* was separated from the ubiquitous promoter, ROSA, by a loxP-flanked neomycin gene, preventing expression of β-galactosidase under normal circumstances. Expression of the cre recombinase by transfecting DNA caused transfected cells to excise the neomycin gene and permanently switch on β-galactosidase expression driven by ROSA. While this study provides convincing evidence that Langerhans' cells were transfected and then migrated to the draining lymph nodes, they again only provided an indirect and coincidental link between antigen expression and presentation to T cells. In this case, presentation could have been achieved by other DC types that captured antigen from the migrating Langerhans' cells.

Our own studies (77) and those of Zhao *et al.* (101) examining the response to HSV have provided evidence that despite the infection of skin or mucosal epithelia by HSV, Langerhans' cells are not responsible for priming virus-specific CTLs. By using the fact that Langerhans' cells but not other DCs are radioresistant, we were able to show that CTL priming depended on a radiosensitive cell type, further identified as a CD8 DCs, despite distinct infection of the skin epithelia by HSV (77). These studies raised the possibility that Langerhans' cells may only prime immune responses under specific and limited conditions or that they simply transport antigen, without playing a direct role in presentation for T-cell priming.

Dermal DCs of the skin or the interstitial DCs of other tissues are largely $CD11b^{+}CD205^{+}CD8^{-}$ (85). They differ from Langerhans' cells in that they do not contain Birbecks granules, and they express lower levels of CD205 than Langerhans' cells. These DCs likely consist of more than one type, but for simplicity here we refer to them as dermal/interstitial DCs. Several studies have implicated these DCs in immunity, particularly the dermal DCs (101–104). While their capacity to make specific cytokines or drive T helper 1 (Th1) versus Th2 immunity is not well documented, we have found that they efficiently cross-presented soluble antigen but not cell-associated antigens (Behrens *et al.* manuscript submitted).

Dermal DCs were recently implicated as the critical cell type for inducing CD4 T-cell immunity to protein antigen injected intradermally (104). Although DCs that appeared to capture antigen in the draining lymph node presented this antigen in a first wave of T-cell stimulation beginning at 4 h, this occurrence was not sufficient to induce sustained T-cell priming and expansion, which required a second wave of DCs that migrated from the site of immunization, arriving 24 h after antigen administration. These cells expressed CD11b and were CD205 intermediate, indicating their derivation from dermal DCs and not Langerhans' cells.

In another study examining the DCs responsible for priming helper T-cell immunity to HSV-2 after vaginal infection, Zhao *et al.* (101) showed that $CD11b^{+}$ subset of submucosal DCs, but not Langerhans' cells, were responsible for initiating immunity. Similarly, two studies examining the response to *Leishmania major* injected intradermally (102, 103) indicated that $CD11b^{+}$ DCs, probably of dermal origin, played an important role in immunity.

For the three blood-derived DC subsets, only the CD8 DCs have a well-documented immune function (as outlined below); the roles of CD4 DCs and $CD4^{-}CD8^{-}$ DCs are poorly understood. There is some evidence that $CD4^{-}CD8^{-}$ DCs preferentially produce IFNγ (105), while no dominant cytokine production has been attributed to the CD4 DCs. The CD4 DCs and $CD4^{-}CD8^{-}$ DCs make up the $CD8^{-}$ splenic DC group,

which has been implicated in Th2 responses (106), but this alignment is not well apportioned. There is evidence that CD8 DCs, which are the $CD205^+$ splenic subset, predominate in T-cell areas of lymphoid organs (107–109), particularly for the spleen, residing in the periarterial lymphoid sheaths. The CD4 DCs and the $CD4^-CD8^-$ DCs, in contrast, are found largely in the marginal zone of the spleen under steady state, non-infectious conditions, though they move to T-cell areas upon exposure to activating stimuli such as lipopolysaccharide (LPS) (109).

The CD8 DCs are the predominant producers of IL-12 (107), an important T-cell stimulatory cytokine, especially for CD8 T-cell proliferation. IL-12 has recently been referred to as the third signal, stressing its importance in T-cell priming versus tolerance (110). Perhaps, the most important property of CD8 DCs with respect to this review is their central role in cross-priming (11) and cross-tolerance (64, 111), which is examined in more detail in the next section.

Cross-presentation of cellular antigens by DC subsets

Cellular antigens are a major source for cross-presentation and can be associated with virus infection, the development of tumors, or immunization with DNA. They can also be used as a source of antigens present in normal tissues for induction of self-tolerance. Several studies have attempted to identify the cells responsible for presentation of cellular antigens, and there seems to be two main DC subsets implicated, the CD8 DCs and the dermal/interstitial DCs. Evidence for the latter, however, entirely relates to presentation of MHC class II-restricted cellular antigens to CD4 T cells, and so it is not strictly cross-presentation. Thus, CD8 DCs are implicated as the major subset responsible for cross-presentation of cellular antigens to CD8 T cells, but the dermal/interstitial DCs may be relevant to this process.

The first definitive study of DC subset involvement in cross-presentation of cellular antigens came from den Haan *et al.* (11), who determined which DC subset was responsible for cross-priming CTL immunity to OVA-loaded spleen cells. In this case, OVA acted as a surrogate intracellular protein antigen and was shown to be cross-presented strictly by the CD8 DC subset (*Table 1*). In related studies, Inaba and coworkers (14) confirmed this finding and extended it to show that CD4 T cells responding to OVA-loaded spleen cells also recognized antigen on CD8 DCs. Townsend *et al.* (112) similarly showed that B cell-derived antigens could be presented to CD4 T cells by CD8 DCs. From these studies, it appeared that intravenously introduced cellular antigens were cross-presented by CD8 DCs, which also presented these antigens to CD4 T cells. Demonstration that cross-priming required that the same DC present antigen to both CD4 and CD8 T cells (113), in a process referred to as cognate help, supports the view that CD8 DCs must present intravenously introduced cellular antigens to both T-cell subsets.

An observation of several studies is that CD8 DCs preferentially capture apoptotic cells (14, 58, 112), which they are then able to cross-present to CD8 T cells (14, 58). These studies suggest that the ability to capture such antigens dictates whether cross-presentation occurs, arguing that all DC subsets possess the machinery to cross-present and will do so as long as they can endocytose the particular antigen. Our own observations do not agree with these conclusions, as we find that when the three subsets of splenic blood-derived DCs (CD4 DC, CD8 DC, and $CD4^-CD8^-$ DC) are exposed to cellular antigens *in vitro*, all three subsets present cell-derived antigens to CD4 T cells, but only the CD8 DCs cross-present such antigen to CD8 T cells (114). These findings led us to conclude that all three DC subsets can capture cellular antigens, but the ability to cross-present is differentially regulated within the subsets. In either case, CD8 DCs are the primary subset responsible for cross-presentation of intravenously introduced cellular antigens that access the spleen.

Examination of the DC subsets involved in cross-presentation of cellular self-antigens expressed by pancreatic islet β cells showed similarly that CD8 DCs cross-presented such antigens derived from normal tissues (64). In these studies, we transgenically expressed enhanced yellow fluorescent protein together with two CD8 T-cell epitopes under the control of RIP in B6 mice, and we were able to detect strong fluorescence by the transgenic islets (*Fig. 2*). Such expression led to cross-presentation of the linked T-cell epitopes in the draining lymph node of the pancreas. This presentation was able to induce proliferation of specific CD8 T cells that were subsequently deleted by an apoptotic mechanism shown in another series of studies to be dependent upon expression of bim in the T cells (62, 65). These studies indicated that, as well as inducing cross-priming, CD8 DCs could induce self-tolerance via cross-presentation of tissue-derived self-antigens. This process has been referred to as cross-tolerance.

In a series of related studies, Hugues *et al.* (115) reported that antigens released from non-obese diabetic (NOD) mouse islets after damage by streptozotocin treatment were presented to CD4 T cells by a $CD11b^+$ DC subset that could induce regulatory T cells. Because CD8 DCs do not express CD11b,

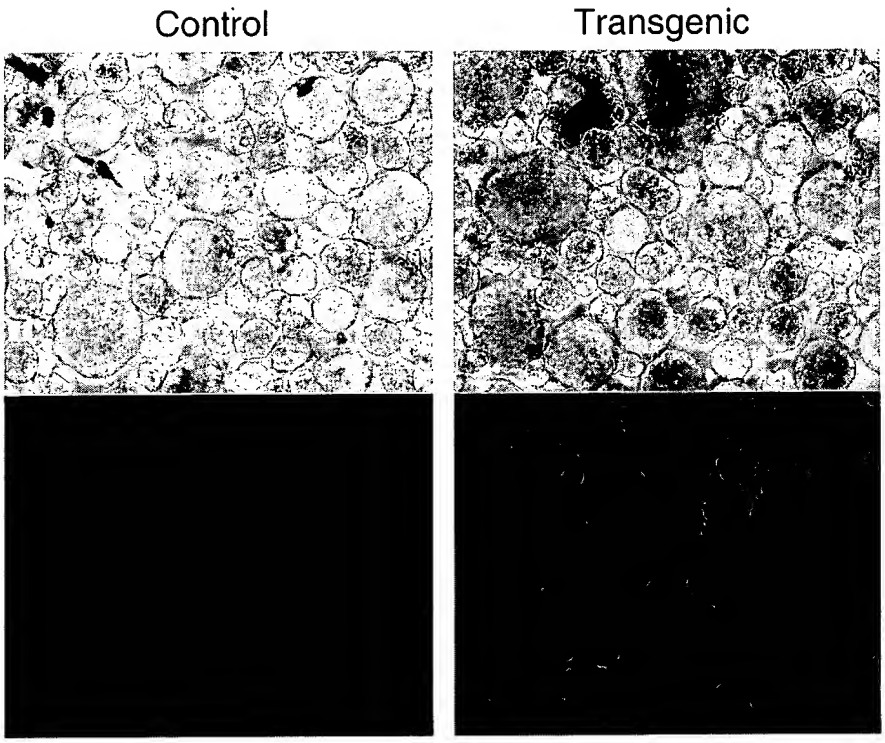

Fig. 2. Expression of enhanced yellow fluorescent protein by isolated islets from transgenic mice expressing this protein linked to two major histocompatibility complex (MHC) class I T-cell epitopes in the β cells of the islets under the control (left) of the rat insulin promoter (right) (64).

this study suggested that different subsets were involved in these two models, either because of genetic differences between NOD (113) and B6 (64) mice or because different DC subsets are responsible for presentation of cellular antigens to CD4 and CD8 T cells. Recently, the Mathis group (114) reported that apoptotic cellular antigens injected into the pancreas of NOD mice were largely transported to the draining LNs by a $CD11b^+$ DCs, consistent with the findings of Hugues *et al.* (115), though in this same study, some CD8 DCs in the pancreatic lymph node were also found to contain antigen, consistent with an additional role for this subset.

These observations for pancreatic antigens are reminiscent of studies by Germain and associates (111) examining antigen trafficking from the gut to the gastric lymph node. While a $CD8^-$ DC was seen to capture cellular material from the stomach, both $CD8^-$ and $CD8^+$ DCs presented the stomach-derived self-antigens to CD4 T cells in the draining gastric LN. Neither the study of dead cell traffic from the NOD pancreas (115) nor the observed presentation of BALB/c gastric antigens (111) examined presentation to CD8 T cells, so cross-presentation was not strictly measured.

We speculate that a $CD11b^+$ subset of DCs (the dermal/interstitial DCs) are responsible for cellular antigen transport and presentation to CD4 T cells, but this subset is not able to cross-present tissue-derived antigens to CD8 T cells. For this activity, they must hand off antigen to the CD8 DCs. *In vitro* examination of cross-presentation of cell-associated OVA by lymph node DC subsets shows that the CD8 DCs are primarily responsible for this function, with $CD11b^+$ dermal DCs unable to cross-present cellular material (Behrens *et al.* manuscript submitted). Consistent with the idea that CD8 DCs receive their antigen from a $CD11b^+$ dermal/interstitial DCs, neither Germain's group (111) nor Mathis' group (116) could find CD8 DCs in the tissues, despite the presence of antigen in this subset within the draining lymph nodes.

Examination of the presentation of HSV antigens provides clear evidence that despite infection of epithelial cells of either the skin or vagina, antigen presentation was not mediated by the predominant DC in this site, i.e. the Langerhans' cells (77, 101). In the vagina (101), a $CD11b^+$ subset of submucosal DCs was shown to present HSV-2 antigens to CD4 T cells, whereas for HSV-1 infection of the skin (77), CD8 DCs were shown to present viral antigens to CD8 T cells. Because CD8 T-cell responses were not measured in the HSV-2 study (101) and CD4 T-cell responses were not measured in our analysis of HSV-1 (80), it is difficult to directly compare findings. However, these findings are consistent with the view that tissue-derived $CD11b^+$ DCs traffic with cellular antigen to the draining lymph node where they present these antigens to CD4 T cells and also supply them to CD8 DCs for cross-presentation to CD8 T cells.

Examination of the uptake of apoptotic cells by splenic DCs provides further support for this view. Morelli *et al.* (117) showed that when fluorescently labeled apoptotic cells were

injected intravenously into mice, $CD11b^+CD8^-$ marginal zone DC captured antigen within the first few hours, but CD8 DCs contained the bulk of antigen at 24–48 h. While these authors favored the idea that $CD11b^+CD8^-$ DCs actually converted into $CD11b^-CD8^+$ DCs, there was no evidence to exclude antigen transfer, which they also suggest as a possibility. Interestingly, in studies examining the presentation of virus-like particles by DC subsets (118), a $CD11b^+CD8\alpha^-$ DC that initially presented these particles was shown to acquire CD8α but not CD8β within 15 h of antigen capture. Because mRNA for CD8α was absent, despite positive surface staining, these findings are consistent with transfer of some surface molecules from CD8 DCs to the $CD11b^+$ subset. Interaction is, therefore, implied between these two DC subsets after introduction of antigen to the system. Perhaps, this interaction is associated with antigen transfer.

When we extended our analysis to viral infections of the lung, further support emerged for the idea that one DC traffics with cellular material from peripheral tissues and passes it on to CD8 DCs in the draining lymph node for cross-presentation. Lung infection with influenza virus or HSV-1 led to antigen presentation by both a $CD8^-$ and a $CD8^+$ DC subset. By administration of carboxyfluorescein diacetate succinamidyl ester (CFSE) to the lung at the time of infection, it was possible to show that the $CD8^-$ DCs presenting antigen trafficked from the lung, while the $CD8^+$ DCs were lymph node resident (Belz *et al.* manuscript submitted). In this case, we could not distinguish whether presentation by $CD8^+$ DCs was due to direct infection or occurred via cross-presentation, but our findings are consistent with the idea that lymph node-resident $CD8^+$ DCs capture cellular antigens from a trafficking $CD8^-$ DC subset and cross-present it to naïve CD8 T cells.

Whether CD8 DCs capture antigen from trafficking DCs or obtain it by other indirect methods from the tissues, e.g. exosomes or HSPs, it is clear that when MHC class I-restricted responses to cellular antigens are examined, CD8 DCs are the major subset responsible for this process and, in many cases, achieve this response by cross-presentation.

What dictates cross-priming versus cross-tolerance?

As CD8 DCs have been shown to participate in both cross-priming and cross-tolerance to cellular antigens, it seems likely that precisely the same DC subset contributes to both immunity and tolerance. However, the existence of two distinct CD8 DC subsets, one tolerogenic and the other immunogenic, cannot strictly be excluded. Whatever the case, specific factors must dictate whether cellular antigens induce immunity or tolerance. The simplest explanation is that CD8 DCs induce tolerance unless exposed to some form of inflammatory stimuli normally associated with infection. Two general forms of inflammatory signals have been postulated (119–122), and both are to some degree supported by experimental evidence. These signals are (i) microbial products such as LPS, double-stranded RNA, and flagellin, which activate DCs via surface receptors like the TLRs, and (ii) endogenous danger signals such as IFNα (94, 123), HSPs (44), and even uric acid (124), which have also been reported to cause DC maturation, converting them into highly stimulatory cells.

For DNA vaccination, which may or may not occur by cross-priming (see next section), the induction of DC maturation is likely to be important. The presence of immunostimulatory CpG motifs has been reported to be critical for induction of DNA-mediated immunity (125, 126). These immunostimulatory CpG motifs appear to signal through TLR9 (127), and they are thought to act by causing maturation of DCs (128, 129). In a recent report, however, both TLR9-deficient mice as well as MyD88-deficient mice were able to generate equivalent CTL responses to that of wildtype mice when injected with DNA containing CpG motifs, despite the failure of DCs from these mice to mature upon exposure to such DNA (130). It is notable, however, that these authors did not examine the maturation of CD8 DCs, leaving open the possibility that this subset is activated by CpG-containing DNA via a pathway independent of TLR9 and MyD88. These findings further suggest that the immunostimulatory nature of CpG motifs associated with DNA may occur by yet other undefined mechanisms, at least for CTL priming.

As an alternative source of immune activation, damage caused by the introduction of DNA into tissues might release intracellular products known to facilitate inflammation, DC maturation, and CTL priming. Consistent with this idea, gene gun vaccination has been shown to increase the number of DCs in the draining lymph node, even when DNA is not associated with the introduced gold particles (131). The DC-stimulating product associated with tissue damage has, however, not been directly identified. As discussed earlier, HSPs can provide an antigen source for cross-priming, but they have also been reported to activate DCs (132). Thus, purified HSPs are thought to generate efficient CTL responses, because they represent both an efficient antigen source and an immunostimulatory signal. In a recent report, however, Rock and colleagues (124) examined the nature of immunostimulatory material from lysed cells and did not find HSPs to be the source of activity. These authors identified uric acid as the cellular component responsible for activation of the immune system. Interestingly, free uric acid did not turn out to be the

active component, but formation of sodium urate crystals was necessary for stimulation of DC maturation and initiation of CTL immunity. The inflammatory nature of sodium urate has been long known by its well-defined role in gouty arthritis, supporting the concept that this compound is immunostimulatory. These authors propose that release of locally high concentrations of uric acid during cell damage leads to crystal formation and the maturation of local DCs.

As highlighted in the former section, DCs like dermal/interstitial DCs or Langerhans' cells may be responsible for transporting cellular antigens into the draining lymph nodes for cross-presentation by CD8 DCs. This role raises the possibility of a two-step scenario for inducing maturation of the CD8 DCs. First, the transporting DCs may mature in response to inflammatory signals, allowing them to prime naïve CD4 T cells. Then these activated CD4 T cells may induce maturation of the CD8 DCs for cross-priming. Consistent with this idea, we have recently demonstrated that injection of activated helper CD4 T cells into mice expressing OVA as a model autoantigen in the pancreas efficiently induced naïve CD8 T cells to cause autoimmune diabetes, though they normally undergo deletional tolerance in the absence of help (114). Thus, provision of helper T cells, already activated by other stimuli, may be enough to convert a tolerogenic response by the CD8 DCs into immunity (in our case autoimmunity).

Cross-presentation and DNA vaccination

In this section, we address the role of cross-presentation in DNA vaccination. Several studies have indicated that a bone marrow-derived cell is required for priming, both for intramuscular injection of plasmid DNA (133, 134) and for cutaneous gene gun-mediated administration (135). By using severe combined immunodeficiency disease (scid) mice as recipients of T cells (as part of a mixed population of spleen cells) and bone marrow cells (as a source of APCs), Doe *et al.* (133) showed that CTL generation by intramuscular injection of DNA encoding viral epitopes required matching the MHC haplotype of the APCs and T cells. Because these authors could inject the APCs and T cells up to 21 days after injection of DNA, their results further implied that the APCs responsible for antigen presentation did not have to be directly transfected with the DNA but could capture antigen from other transfected cells. Corr *et al.* (134) used parent into F1 chimeras to more directly show that priming was mediated by a bone marrow-derived cell. In this case, H-2^{bxd} mice were lethally irradiated and reconstituted with either H-2^{b} or H-2^{d} bone marrow. When these chimeras were primed intramuscularly with plasmid DNA encoding both an H-2^{b}- and an H-2^{d}-restricted epitope, the induced CTL responses were restricted to the MHC haplotype of the donor bone marrow. Interestingly, when gene gun vaccination was examined for its dependence on bone marrow-derived cells (135), similar results were observed. In these studies, Iwasaki *et al.* (135) used the same parent into F1 chimera strategy to demonstrate gene gun priming depended on presentation by a bone marrow-derived cell. The interesting point here is that given our recent knowledge that Langerhans' cells are not bone marrow-derived in such chimeras (136), the observations of Iwasaki *et al.* (135) imply that Langerhans' cells are not responsible for CTL priming after gene gun inoculation of DNA.

Various studies (131, 137–139) have reported that DCs can be directly transfected with DNA, particularly using gene gun administration where skin-derived DCs (138, 139), including Langerhans' cells (100), appear to be transfected. In some studies (131, 137), DCs directly expressing transfected antigens were implicated in priming. These reports relied on evidence that antigen presentation could be depleted using antibody depletion techniques with antibodies specific for cell surface molecules encoded by the immunizing DNA. Unfortunately, subsequent studies have demonstrated the exchange of surface membrane molecules from DC to DC (42, 118), raising the possibility that this approach might not exclude presentation by cross-presenting DCs. While Garg *et al.* (100) showed that Langerhans' cells expressing transfected DNA persisted in the draining lymph node for about two weeks, they did not formally show that it was the Langerhans' cells that were responsible for the coincident antigen presentation. Thus, while these cells may have carried the antigen into the lymph node, it is possible they passed this antigen to other DCs for cross-presentation.

There is relatively limited evidence that cross-presentation is important for DNA vaccination, but equally, those studies implicating direct priming are not completely convincing. As indicated earlier, Doe *et al.* (133) implicated cross-priming in their scid model, as CTLs were induced even when T cells and APCs were adoptively transferred up to 21 days after DNA administration. As it was highly unlikely that DNA could survive for this time and then transfect incoming APCs, the only logical conclusion was that previously transfected scid cells supplied antigen to donor APCs. In a later series of studies, Corr *et al.* (140) provided evidence for cross-priming using a complex DNA priming model, which could be repressed by the presence of tetracycline. These authors showed that if mice were injected intradermally with DNA

in the presence of tetracycline repression for one week, and then the skin at the inoculation site removed and tetracycline administration stopped, no priming occurred. Because mice with intact skin showed efficient priming after tetracycline withdrawal, this finding implicated cells within the skin as the antigen source. They also showed that CTL responses were greater when non-bone marrow-derived cells were able to express plasmid-derived antigens.

Cho *et al.* (141) used tissue-specific promoters to express gene gun-administered DNA in cells of dendritic/macrophage origin or keratinocytes. While expression in either tissue type was able to induce CTLs, responses were shown to be more rapid and sustained when expression was restricted to keratinocytes. These findings implied that cross-priming was more efficient than direct priming. Unfortunately, it is very difficult to quantitate antigen expression *in vivo* for each expression vector, thus leaving open the possibility that differences in antigen expression may explain variations in efficiency.

Co-administration of an apoptosis-inducing gene together with the gene of an antigen has been shown to improve priming by both intramuscular and gene gun injection of DNA (52, 53). This outcome is hard to reconcile with anything other than cross-priming, which is suggested to be capable of targeting apoptotic cells. Interestingly, however, in another study (142), co-administration of anti-apoptotic genes, particularly Bcl-X_L, was shown to improve priming by gene gun-administered DNA. In this case, improved priming was attributed to the extended survival of directly transfected APCs, which are normally relatively short lived. Thus, direct priming rather than cross-priming might have been implicated here.

Because various DNA vaccination studies have implicated direct priming by transfected DCs while others have favored cross-priming, it may appear difficult to draw conclusions as to which mode of priming predominates, particularly considering the highly reputable nature of these studies. Our favored view, however, is that both modes are possible, though under specific circumstances one will predominate over the other. For example, cross-presentation of islet-expressed transgenes has been shown to depend on antigen dose (51, 63). At a certain dose threshold, islet cells might express enough antigen to be directly recognized by CTLs, but insufficient to allow cross-presentation by DCs. As the antigen expression levels are increased, both direct presentation by islets and cross-presentation of islet antigens on DCs can occur. These findings suggest that if DNA-encoded antigens are expressed at a suboptimal level, then cross-presentation may not be possible, though direct presentation could still occur. Such DNA-encoded antigens will only be able to prime by cross-presentation when their expression levels are increased or if the cells expressing these suboptimal concentrations of antigen are killed, for example by co-expression of apoptosis-inducing molecules (52, 53). The latter effectively would be making more antigen available for cross-presentation.

Clearly, tissue-specific expression of DNA-encoded antigens would favor cross-presentation when the specificity is for cells other than DCs (141). Similarly, for ubiquitously expressed high dose antigens, priming may occur largely via cross-presentation. While transfected DCs would contribute to such immunity, the short-lived nature of this cell type (88) compared to that of cells such as keratinocytes may limit their contribution.

We speculate that the sequence of the antigen may influence the contribution of cross-priming. So far, there is little experimental evidence to support this possibility, but perhaps glycoprotein from LCMV, which does not cross-prime even when transgenically expressed in spleen cells (67), is one such example.

Conclusions

Cross-presentation is likely to be important for the generation of CTL immunity to a variety of cellular antigens, including viral, tumor, and DNA-encoded antigens. The mechanistic basis for this process is beginning to be elucidated, suggesting that the specific fusion of ER and phagosomes introduces MHC class I-presenting machinery into close association with phagocytosed antigens. DCs appear to be the major cell type capable of cross-presentation, and they are particularly important for the initiation of immunity. Recent evidence indicates that DCs are not a single cell type, however, but consist of at least six different subsets. Of these, CD8 DCs appear to be the primary subset involved in cross-presentation of cellular antigens. For DNA vaccination, several studies suggest a role for cross-presentation, while others argue that direct presentation by transfected DCs is crucial. Most likely, both mechanisms can be involved, but one predominates over the other depending on the level of antigen expression, the cell type specificity of the expression vector, and other undefined factors relating to the nature of the antigen. Defining these factors and deciphering approaches that can measure the contribution of cross-priming and direct priming without impairing one or the other will be important steps for the future. Similarly, precise knowledge of the role of different DC subsets in each aspect of immunity will be invaluable to our ability to manipulate the immune system in our favor.

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